Hypoxic stress, brain vascular system, and β-amyloid: A primary cell culture study

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This study stresses the hypothesis whether hypoxic events contribute to formation and deposition of β-amyloid (Aβ) in cerebral blood vessels by affecting the processing of endothelial amyloid precursor protein (APP). Therefore, cerebral endothelial cells (ECs) derived from transgenic Tg2576 mouse brain, were subjected to short periods of hypoxic stress, followed by assessment of formation and secretion of APP cleavage products sAPPα, sAPPβ, and Aβ as well as the expression of endothelial APP. Hypoxic stress of EC leads to enhanced secretion of sAPPβ into the culture medium as compared to normoxic controls, which is accompanied by increased APP expression, induction of vascular endothelial growth factor (VEGF) synthesis, nitric oxide production, and differential changes in endothelial p42/44 (ERK1/2) expression. The hypoxia-mediated up-regulation of p42/44 at a particular time of incubation was accompanied by a corresponding down-regulation of the phosphorylated form of p42/44. To reveal any role of hypoxia-induced VEGF in endothelial APP processing, ECs were exposed by VEGF. VEGF hardly affected the amount of sAPPβ and Aβ(1–40) secreted into the culture medium, whereas the suppression of the VEGF receptor action by SU-5416 resulted in decreased release of sAPPβ and Aβ(1–40) in comparison to control incubations, suggesting a role of VEGF in controlling the activity of γ-secretase, presumably via the VEGF receptor-associated tyrosine kinase. The data suggest that hypoxic stress represents a mayor risk factor in causing Aβ deposition in the brain vascular system by favoring the amyloidogenic route of endothelial APP processing. The hypoxic-stress-induced changes in β-secretase activity are presumably mediated by altering the phosphorylation status of p42/44, whereas the stress-induced up-regulation of VEGF appears to play a counteracting role by maintaining the balance of physiological APP processing.

Keywords: Amyloid precursor protein, Primary cell culture, Brain vascular endothelial cell, Tg2576 mouse, VEGF, SU-5416, ELISA

Introduction

Alzheimer's disease (AD), a heterogeneous and multifactorial disorder, is characterized clinically by dementia and pathomorphologically by extracellular accumulation of β-amyloid (Aβ) plaques and intracellular tau-pathology. Aβ peptides are formed through proteolytic processing of the amyloid precursor protein (APP) by subsequent catalytic actions of the β- and γ-secretase. Abnormal APP processing has been assumed to play a causative role in AD development resulting in an imbalance of production and clearance of Aβ (amyloid cascade hypothesis of AD; for reviews, see e.g.[1–5]), while the validity of the amyloid hypothesis has also been questioned in terms of its causality (for review, see e.g.[6]).

However, many AD cases also demonstrate cerebral vascular changes and impaired regulation of cerebral blood flow, that have been assumed to play an important role in AD pathogenesis (vascular hypothesis of AD, for review, see e.g.[1,7–9]). Insufficient cerebral blood flow and hypoperfusion may induce hypoxia-sensitive pathways leading to inflammation with up-regulation of pro-inflammatory cytokines, and to oxidative stress with generation of reactive oxygen and nitrogen species which may be detrimental to vascular integrity and function. Indeed, cerebrovascular abnormalities such as thickening of the microvascular basement membranes, decreased luminal diameter, and microvascular degeneration, in particular in the temporal-parietal cortex, have frequently been observed in AD patients.[9]

The cerebrovascular pathology includes the accumulation of Aβ in blood vessels and microvascular degeneration (cerebral amyloid angiopathy) affecting cerebral endothelium and vascular smooth muscle cells.[9–11] The cerebral amyloid angiopathy which is observed during normal aging, and in the majority of AD cases, is likely caused by the failure of Aβ elimination from the brain parenchyma,[12] while a role of

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vascular smooth muscle and endothelial cells (ECs) by abnormal processing of APP has also been suggested.[13,14]

There are a number of reports that ischemia and hypoperfusion may trigger accumulation and cleavage of APP into Aβ, and its deposition in the brain including the vascular system (for reviews, see e.g.[12,15]), as well as affecting Aβ degrading enzymes like neprilysin and endothelin converting enzyme.[16] The identification of a functional hypoxia-responsive element in the BACE1 gene promoter has disclosed a potential mechanism by which hypoxia up-regulates β-secretase cleavage of APP.[17]

On the other hand, the microvascular degenerations observed in AD may also be the consequence of the vasoactive detrimental effects of Aβ.[18] Aβ is a potent vasoconstrictor in the brain, as has been shown *in vivo* and *in vitro* by application of exogenous Aβ to normal blood vessels and to mouse cortex.[19–23] On the other hand, Aβ may cause degeneration of both the larger perforating arterial vessels as well as cerebral capillaries, presumably mediated through the induction of reactive oxygen species by activation of NADPH oxidase, which may subsequently severely affect regulation of cerebral blood vessels and brain perfusion as well as impair the blood brain barrier.[11,24–26] Currently, in a transgenic AD mouse model with strong cerebral amyloid angiopathy, a particular role of early perivascular astrocytic dysfunction in impairing cerebrovascular and metabolic pathology has been suggested.[27]

As vascular ECs are also capable to express and to secrete APP,[28] this study stresses the hypothesis whether hypoxic events may contribute to formation and deposition of Aβ in blood vessels by favoring the amyloidogenic processing of endothelial APP. To address this hypothesis, primary cerebral ECs derived from transgenic Tg2576 mouse brain, were subjected to short periods of hypoxic stress, followed by assessment of formation and secretion of APP cleavage products such as sAPPα, sAPPβ, and Aβ as well as the expression of endothelial APP.

Materials and methods

Preparation of primary cerebral endothelial cell cultures

Primary cerebral microvascular ECs were isolated from 2-month-old Tg2576 mouse brains according to the method by Ichikawa *et al.*[29]

The Tg2576 mice contained as transgene the human APP695 with the double mutation (K670N, M671L), which was found in a large Swedish family with early onset of AD, as developed and described previously by Hsiao *et al.*[30] The transgeneity of the pups was determined in tail tissue by polymerase chain reaction.[30]

Culture solutions

Standard isolation medium (SIM) represents Ml99 containing 20 mM HEPES, 25 mM sodium bicarbonate, penicillin streptomycin mixture (50 IU/ml, 50 μg/ml), amphotericin B (2.5 μg/ml), heparin (10 U/ml), and 5% FBS, and adjusted to a pH 7.4.

Growth medium-A (GM-A) represents DMEM containing 20 mM sodium bicarbonate, 2 mM L-glutamine, ECGF (150 μg/ml), penicillin–streptomycin mixture (50 IU/ml, 50 μg/ml), amphotericin B (2.5 μg/ml), MC-210 (0.5 μg/ml), heparin (10 U/ml), and 20% FBS and adjusted to a pH 7.4.

Growth medium-B (GM-B) represents DMEM containing 20 mM sodium bicarbonate, 2 mM L-glutamine, penicillin–streptomycin mixture (50 IU/ml, 50 μg/ml), amphotericin B (2.5 μg/ml), ECGF(150 μg/ml), 5% FBS, and 5% donor horse serum and adjusted to a pH 7.4.

Freezing medium-A (FM-A) represents DMEM containing 20 mM sodium bicarbonate, 2 mM L-glutamine, penicillin–streptomycin mixture (50 IU/ml, 50 μg/ml), amphotericin B (2.5 μg/ml), heparin (10 U/ml) and 20% FBS and adjusted to pH 7.4.

Freezing medium-B (FM-B) represents DMEM containing 20 mM sodium bicarbonate, 2 mM L-glutamine, penicillin–streptomycin mixture (50 I.U./ml, 50 μg/ml), amphotericin B (2.5 μg/ml), heparin (10 U/ml), 20% dimethyl sulfoxide, and 40% FBS and adjusted to a pH 7.4.

Isolation method

Ten 2-month-old Tg2576 mice were anesthetized by CO_2 inhalation, the brains were isolated, and cerebelli and primary cerebral stems were removed. The remaining primary cerebral tissue was placed into 70% ethanol, moved into ice-cold PBS (-) and then rinsed five times in ice-cold SIM. The cerebral hemispheres were carefully dissected out and finely minced into small pieces, and then added with serum free SIM (SFSIM). The suspension was centrifuged at 1000 *g*, at 4°C for 10 minutes. The resulting pellet was resuspended in 0.02% dispase solution (in SFSIM) and incubated at 37°C for 60 minutes. The dispase suspension was again centrifuged at 1000 *g*, at 4°C for 10 minutes, and the pellet was resuspended in 15% dextran solution (in SIM). After centrifugation at 4500 *g*, 4°C for 10 minutes, the fat pad and dextran solution were collected into a new tube and centrifuged once again. The two pellets were collected and resuspended in the new dextran solution and centrifuged again. The resulting tissue pellet was resuspended in SFSIM and filtered through a 300-μm mesh. The microvessel suspension was dissociated using 0.1% collagenase/dispase (in SFSIM) at 37°C for 30 minutes. After the enzyme treatment, the microvessels were collected by centrifugation (1000 *g*, 4°C

for 10 minutes), and resuspended in SIM. The suspension was layered onto Percoll gradient formed by centrifugation of 50% isotonic Percoll at 25 000 *g*, at 4°C for 70 minutes, and then centrifuged at 1650 *g*, at 4°C for 10 minutes. After the Percoll gradient centrifugation, three layers could be observed. The EC fragments formed a band around the middle third of the gradient, and the entire middle layer was collected from gradients. The pellet was washed three times in SIM. The preparation was resuspended in GM. The cell suspension was seeded onto collagen-coated 35-mm tissue culture dishes or 24-well tissue culture plates at a density of 50 000 cells/cm^2. The cells were incubated in a moist 5% CO_2, 95% air atmosphere, at 37°C. After the cell attachment, the cells were maintained according to the method of Gordon *et al.*[31] with minor modifications. For the first 3 days, the cells were cultured in GM-A and then cultured in GM-B after day 3. The culture medium was changed every other day until the cells became a confluent monolayer.

Freezing method for the isolated cells

The isolated cells (the pellets from the final centrifugation) were resuspended in FM-A. Aliquots from the cell suspension were placed in cryotubes at 4°C and then an equal volume of FM-B was added to each tube. The cryotubes were stored at −20°C for 24 hours and then moved to an ultrafreezer at −80°C for up to 6 months. For cell culture, the frozen cells were thawed in FM-A and washed three times with GM-A.

The purity of EC cultures was checked by immunostaining with the rabbit anti-von Willebrandt factor (Factor VIII-related antigen) antiserum (Sigma-Aldrich, Munich, Germany, and the immunoperoxidase method was used to visualize the signal.

Treatment of EC culture and tissue preparation

Following three to seven days of culture, the culture medium was replaced by colorless stimulation medium without serum containing the same components like culture medium but supplemented with 4 mM L-glutamine.

For hypoxic stress experiments, the stimulation medium was perfused by nitrogen gas for 30 minutes to reduce the oxygen content in the medium, and immediately used to replace the culture medium of EC. Incubations of cells were continued in the presence of hypoxic colorless medium for up to 48 hours as indicated. EC treated similarly but in the presence of normal (normoxic) medium were considered as control incubation.

For drug treatment experiments, EC were incubated with 800 µl stimulation medium/well in the presence of 1 ng/ml VEGF (R&D Systems, Germany) or 50 µM SU5416 (Biozol, Germany) for six, and 24 hours. Control incubations were treated similarly but in the absence of any drug.

Following incubations, the culture medium was carefully separated from the cell layer, centrifuged at 200 *g* for 10 minutes to remove cell debris. The supernatant was stored at −20°C pending quantitation of VEGF, sAPPβ, sAPPα, and β-amyloid present in the medium.

Cells attached at the bottom of the well, were properly scraped off, homogenized by sonification in 400 µl/well of PBS, divided in aliquots, and stored at −20°C. Aliquots of cell preparations were used for Western blotting, and to assay the protein content as described elsewhere.[32]

Assay of VEGF level

In select experiments, the constitutive secretion of VEGF by primary EC into the medium was assayed by enzyme-linked immunosorbent assay (ELISA) applying a commercial kit, and was performed according to the manufacturer's protocol (R&D Systems, Germany). The amount of VEGF was calculated by comparison with a standard curve, and expressed as pg/ml.[33,34]

To minimize falsifications of the data due to small variations in cell number between wells and between experimental sessions, VEGF values assayed in the medium were referred to the protein content of the particular cell homogenate measured at the end of incubation.

Assay of APP cleavage products

sAPPα and sAPPβ

To quantitate the amount of human APP cleavage products generated in EC and released into the culture medium as a consequence of experimental treatment, commercially available, solid phase sandwich ELISAs (IBL Hamburg, Germany) were applied according to the manufacturer's protocol. The amounts of sAPPα, and sAPPβ were calculated by comparison with a standard curve of recombinant sAPPα, and sAPPβ, respectively, and expressed as ng/ml.[33,34]

To minimize falsifications of the data due to small variations in cell number between wells and between experimental sessions, sAPPα and sAPPβ values assayed in the medium were referred to the protein content of the particular cell homogenate measured at the end of incubation.

Assay of β-amyloid

The amount of human β-amyloid (Aβ) peptides secreted into the cell culture medium during incubation was analyzed using a sandwich ELISA system for Aβ(1–40) commercially obtained from Invitrogen (Darmstadt, Germany), and carried out according to

the manufacturer's protocol. The amount of Aβ(1–40) was calculated by comparison with a standard curve of synthetic human Aβ(1–40), and expressed as pg/ml.[33,34]

To minimize falsifications of the data due to small variations in cell number between wells and between experimental sessions, Aβ(1–40) values assayed in the medium were referred to the protein content of the particular cell homogenate measured at the end of incubation.

Western blot analysis to detect APP

Proteins of EC preparations were separated using standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 10% polyacrylamide-gel), minigels (Miniprotean; Bio-Ra, Munich), subsequently, transferred to a nitrocellulose-membrane (Protean BA85, pore size 45 μm; Schleicher&Schüll) using a Bio-Rad tankblot system. Membranes were blocked for 60 minutes at room temperature with 4% bovine serum albumin in Tris-buffered saline containing 0.1% Tween 20 (TBST) and incubated with the primary antibodies over night at 4°C. The following primary antibodies were applied: the monoclonal antibodies anti-β-APP (22C11; Chemicon International, 1:2500) to detect APP, and the anti-mouse β-actin (Sigma, Munich, Germany, 1:10 000) to be used as internal standard. The blots were washed three times for five minutes with (TBST). The secondary antibody conjugated with horseradish peroxidase (goat-anti-mouse) was incubated for 60 minutes at room temperature, then washed three times with TBST. Protein bands were visualized by enhanced chemiluminescence detection (Western Blotting Luminol Reagent; Santa Cruz Biotechnology, Heidelberg, Germany), according to the manufacturer's protocol.

Immunoblots were quantitatively evaluated by computer-assisted densitometry using the software package TINA (RAYTEST, Berlin). The optical density level of each protein band of interest was normalized to the corresponding expression level of β-actin. Normalized APP expression data are expressed as percentage of corresponding normoxic controls.[34]

Statistical analysis

All data were presented as mean ± SEM. Statistical comparison between experimental groups were tested using one-way analysis of variance, followed by a Student's *t*-test analysis using SPSS software. *P* values <0.05 were considered as statistically significant.

Results

Hypoxic stress affects expression and processing of APP

To reveal whether hypoxic stress affects the metabolism of APP, the cleavage products of APP such as sAPPβ, sAPPα, and Aβ(1–40) released into the medium of cultured EC were quantified by ELISA after varying incubation times.

sAPPβ

Already in control incubations (normoxic conditions), significant amounts of sAPPβ are released by EC, in an incubation time-dependent manner (data not shown). However, exposure of primary EC by hypoxic stress resulted into considerably enhanced formation and secretion of sAPPβ into the culture medium as compared to corresponding values assayed under normoxic control conditions (Fig. 1). Already 6 hours after hypoxic stress of EC the amount of sAPPβ released into the culture medium was higher by about 80% ($P < 0.01$) as compared to that detected under normoxic conditions. Following an incubation period for up to 24 hours the amount of sAPPβ detected in the culture medium was still higher by 160% ($P < 0.01$) over control, with no further increase till 48 hours of incubation (Fig. 1).

sAPPα

In control incubations (normoxic conditions), sAPPα is released by ECs, in an incubation time-dependent manner (not shown). However, hypoxic stress led to

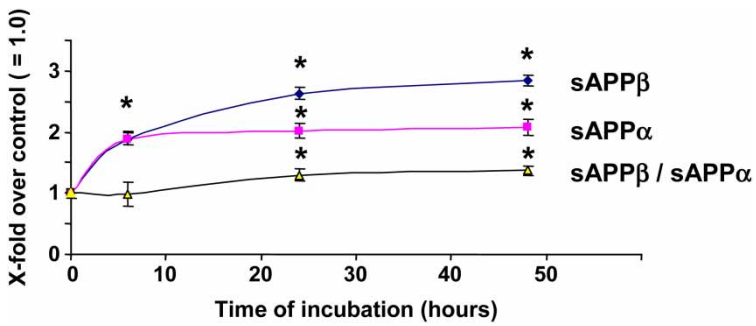

Figure 1 Effect of hypoxic stress of primary EC on formation and secretion of sAPPβ, and sAPPα into the culture medium. The ratio of corresponding amounts of sAPPβ and sAPPa is plotted against the incubation time. Data are expressed as x-fold of control (= 1.0; incubations in normoxic condition), and represent the mean ± SEM of six separate experiments. *$P < 0.05$ vs. control, two-tailed Student's *t*-test.

enhanced generation and release of sAPPα into the culture medium by about 100% ($P < 0.05$), already detectable after a 6 hour-incubation period, with no further increase for up to 48 hours of incubation (Fig. 1).

Ratio of sAPPβ and sAPPα

To reveal whether hypoxic stress may shift the route of APP processing toward the amyloidogenic one, the ratio of the amount of sAPPβ and sAPPα released into the culture medium is plotted versus incubation time. As shown in Fig. 1 hypoxic stress drives the endothelial APP processing toward the amyloidogenic route.

APP expression

To disclose whether the hypoxic stress-induced changes in endothelial APP processing are accompanied by alterations in APP expression, samples of cell homogenates of primary EC were subjected to semiquantitative Western analysis for APP at the end of the incubation period. Representative examples of the immunoblots are shown in Fig. 2A. Densitometric evaluation of the blots demonstrated that hypoxic stress gradually increased the level of APP expression with incubation time. Following 24 hours of incubation, the expression level of cellular APP in hypoxia-stressed EC was significantly increased by 88% ($P < 0.05$) as compared to cells incubated under normal conditions (Fig. 2B).

Hypoxic stress induces VEGF up-regulation and secretion into the culture medium

EC are known to respond to hypoxic, ischemic, or hypoglycemic stress by up-regulation of VEGF. To reveal whether the primary EC used in this study are physiologically functional and are capable to respond to hypoxic stress, the formation and secretion of VEGF into the culture medium was assayed by ELISA. Indeed, as shown in Fig. 3, after a delay of 4 hours post-hypoxia, ECs started to secrete VEGF

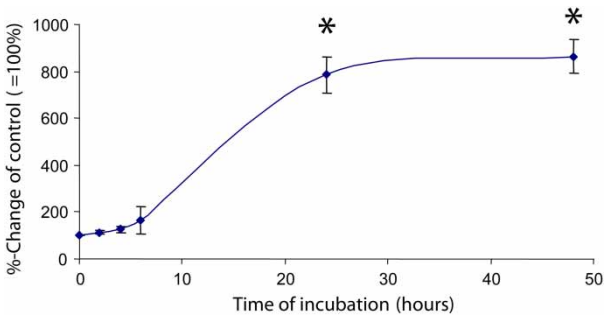

Figure 3 Effect of hypoxic stress with increasing incubation time on up-regulation and secretion of endothelial VEGF into the culture medium. Data are expressed as percentage change of control (= 100%; normoxia), and represent the mean ± SEM three separate experiments. *$P < 0.05$ vs. control, two-tailed Student's *t*-test.

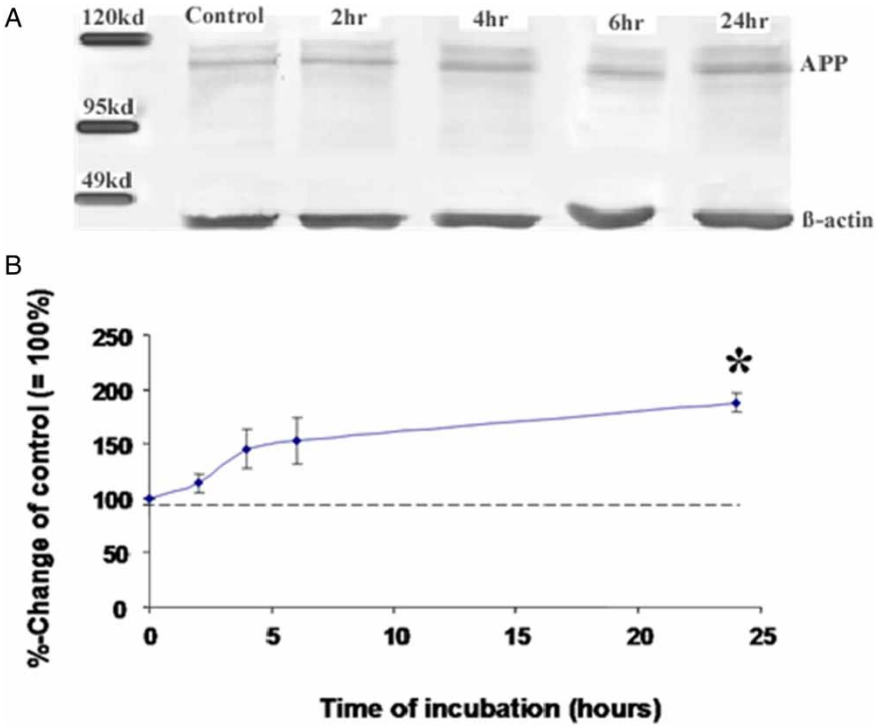

Figure 2 Effect of hypoxic stress on APP expression in primary EC with increasing incubation time. (A) Samples of cell homogenates were subjected to SDS-PAGE, and APP was identified by western blotting using anti-APP monoclonal 22C11 antibody. β-actin was used as internal control to normalize APP expression data. (B) Results of densitometric analysis of immunoblots for APP. Normalized APP expression is expressed as percentage change of corresponding normoxic controls and represents the mean ± SEM of six experiments. *$P < 0.05$ vs. control, two-tailed Student's *t*-test.

into the culture medium. The amount of VEGF released into the culture medium, strikingly increased with further incubation, peaking at 24 hours and persisting at this level for up to 48 hours post-hypoxia, as compared to corresponding normoxic control incubations.

Effect of VEGF on endothelial APP processing

In brain slices and primary neuronal cell cultures VEGF has been shown to affect APP processing.[33,34] Thus, the question arose whether the hypoxia-induced up-regulation of VEGF is inducing and/or mediating the changes of endothelial APP processing following hypoxic stress.

Six hours after incubation of primary EC in the presence of 1 ng/ml VEGF the amount of sAPPβ assayed in the culture medium was lower by about 25% ($P < 0.05$), as compared to that detected in corresponding control medium (Fig. 4). However, following exposure of EC for up to 24 hours by VEGF the amount of sAPPβ accumulated in the incubation medium was no longer different to that detected in culture medium of control incubations in the absence of any drug (Fig. 4). Similarly, the exposure of EC by VEGF for up to 24 hours did not affect the amount of Aβ(1–40) secreted into the culture medium in comparison to control incubations in the absence of any drug (Fig. 4).

The effect of VEGF on APP processing is presumably mediated through VEGF receptors which mediate their actions through activation of the VEGF receptor-associated tyrosine kinase.

To disclose whether the effect of VEGF is mediated by VEGF receptor associated tyrosine kinase, primary EC were incubated in the presence of the tyrosine kinase inhibitor SU-5416 for various periods of time followed by assessment of sAPPβ and Aβ(1–40) accumulated in the culture medium.

Six hours after incubation of primary EC by 50 μM SU-5416 the amount of sAPPβ assayed in the culture medium was lower by about 24% ($P < 0.05$), as compared to that detected in corresponding control medium (Fig. 4). However, following exposure of EC for up to 24 hours in the presence of SU-5416, the amount of sAPPβ accumulated in the incubation medium was no longer different to that detected in culture media of control incubations in the absence of any drug (Fig. 4). In contrast, treatment of EC with SU-5416 for 6 hours resulted in decreased secretion of Aβ(1–40) into the culture medium by 38% ($P < 0.01$), while further SU-5416 exposure of EC for up to 24 hours led to even higher reduction of Aβ(1–40) secretion into the culture medium (by 66%, $P < 0.001$; Fig. 4).

Effect of hypoxic stress on MAPK signaling in EC

The data obtained in chapter 3.3 provide evidence that VEGF alone does not account for the hypoxia-mediated effects on APP processing. Conversely, VEGF appears to play a counteracting role by maintaining a balanced APP processing.

On the other hand, MAPK signaling may also be involved in the hypoxia-induced effects on APP cleavage. To disclose whether the changes in APP expression and processing induced by hypoxic stress are interrelated with intracellular MAPK signaling cascades, cell homogenates of primary EC were subjected to SDS-PAGE, blotted, and immunostained for key enzymes of MAPK signaling cascades (p42/44, p38, and JNK), including their phosphorylated forms.

In Fig. 5A representative examples of western blots from cell homogenates of EC are shown which were

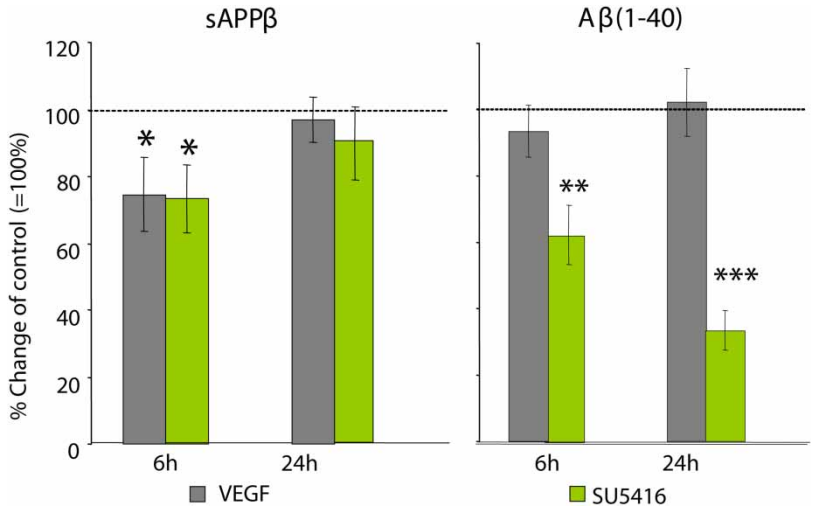

Figure 4 Effect of exposure of endothelial cells by either 1 ng/ml VEGF or 50 μM SU-5416 on secretion of human sAPPβswe and Aβ(1–40) into the culture medium analysed by commercial ELISA-Kits. Data are expressed as percentage change of control (= 100%; incubations in the absence of VEGF or SU-5416), and represent the mean ± SEM of five to six separate experiments.

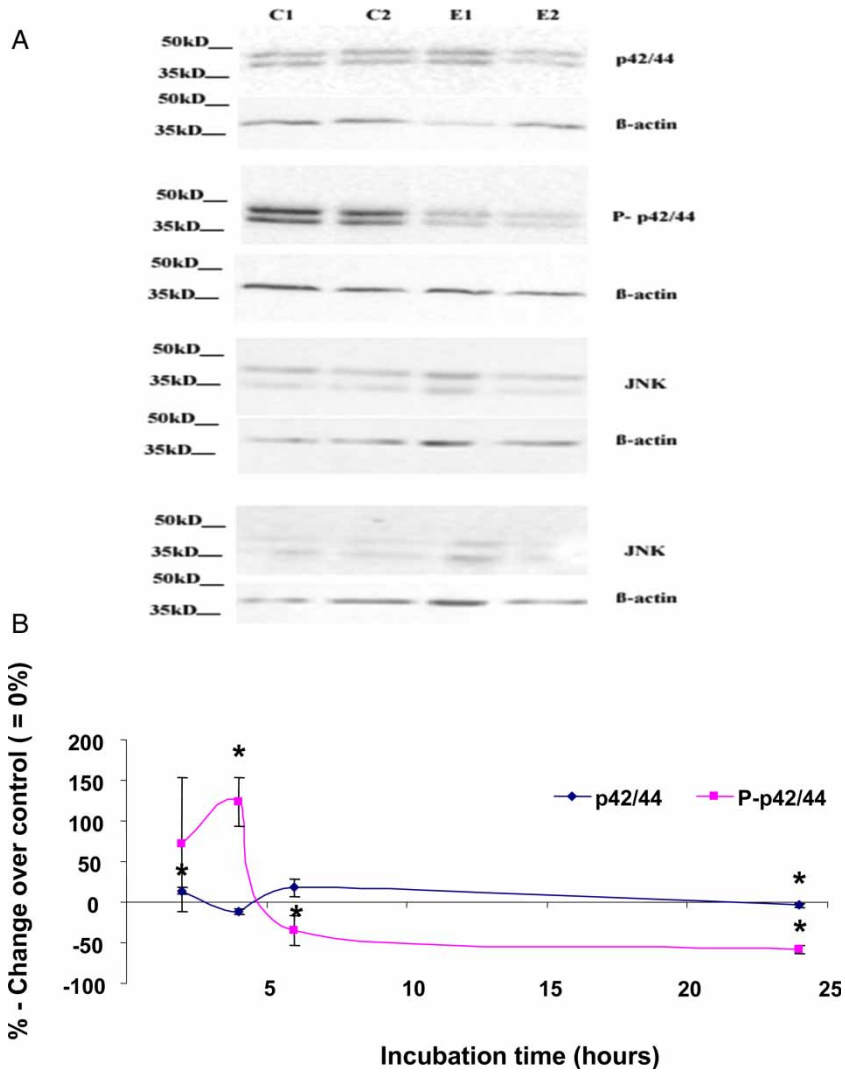

Figure 5 (A) Representative examples of western blots of cell homogenates of primary endothelial cells which were stained with antibodies against p42/44, phospho-p42/44 (P-p42/44), JNK and phospho-JNK (P-JNK). Data were normalized against β-actin as internal protein control. B. Densitometric evaluation of immunoblots stained for p42/44 and P-p42/44 obtained from hypoxic-stress-exposed EC preparations at the end of incubation times indicated. Data are expressed as a percentage change (up-/down-regulation) of the corresponding normoxic controls and represent mean ± SEM of three experiments. *$P < 0.05$ vs. control, two-tailed Student's *t*-test.

stained with antibodies against p42/44, phospho-p42/44 (P-p42/44), JNK and phospho-JNK (P-JNK). The expression of p38 could not be detected in the primary EC preparations used in this study.

The results of the densitometric evaluation of the immunoblots are graphed in Fig. 5B. For each protein studied the expression level presented as hypoxic stress-mediated up/down-regulation, is plotted against the corresponding time of incubation (Fig. 5B).

The densitometric evaluation of the immunoblots revealed that the exposure of EC to hypoxic stress affected neither the expression of phosphorylated nor of non-phosphorylated forms of JNK as compared to the corresponding normoxic controls, regardless of the incubation time examined (data not shown). In contrast, the expression of p42/44 and P-p42/44 were found to be differentially affected when EC were subjected to hypoxic stress as compared to corresponding control incubations (Fig. 5B). When comparing the incubation-time dependent expression pattern of p42/44 and P-p42/44, opposing expression profiles over the incubation time are observed: hypoxia-mediated up-regulation of p42/44 at a particular time of incubation was accompanied by a corresponding down-regulation of P-p42/44 (Fig. 5B). During the first 4 hours of incubation the expression of P-p42/44 was strikingly up-regulated by 124% ($P < 0.05$), followed by down-regulations by −25%, and −58% ($P < 0.05$) at 6 and 24 hours of incubation, respectively. In contrast, the expression of p42/44 was down-regulated by −7% at 4 hours of incubation, but slightly up-regulated by about 20% ($P < 0.05$), and down-regulated by −7% at 6, and 24 hours of incubation, respectively (Fig. 5B).

Discussion

The origin of the vascular amyloid deposits observed in most cases of AD is still controversial, neuronal versus vascular. To address this point, this study was undertaken to disclose whether hypoxic stress may contribute to the cerebral amyloid angiopathy[10,13] by affecting endothelial APP processing. Therefore, primary cerebral EC derived from transgenic Tg2576 mouse brain, were subjected to short periods of hypoxic stress, followed by assessment of formation and secretion of APP cleavage products such as sAPPα, sAPPβ, and Aβ. The main finding of this study is that hypoxic stress changes the ratio of α-, and β-secretory processing of endothelial APP by favoring the amyloidogenic route of APP metabolism, which is accompanied by induction of VEGF, generation of NO, and changes in p42/44 (ERK1/2) expression.

The primary ECs used in this study were obtained from brain tissue of 2-month-old transgenic Tg2576 mice. The Tg2576 mouse brain demonstrates about a six-fold higher expression of the human APP transgene as compared to the endogenous murine APP,[30] which makes assessments of APP cleavage products more accessible, and allows to exploit the greater availability of antibodies directed against human APP cleavage products.

The purity of the isolated EC was checked by subjecting sample preparations to immunocytochemistry using an antiserum directed against the Von-Willebrandt Factor (Factor VIII-related antigen), a reliable marker for EC. Cross-checking revealed that the primary EC cultures used for the experiments did not contain significant amounts of neurons and/or astrocytes. The ECs responded to hypoxic stress by up-regulation of VEGF and production of reactive nitrogen species indicating their viability and proper physiological function.

Incubation of EC under normoxic conditions (controls) for up to 24 hours resulted already in an incubation time-dependent accumulation of sAPPα, sAPPβ and Aβ peptides in the culture medium. The release of APP cleavage products by EC indicates a continuing proteolytic processing of cellular APP. The ratio of the amount of sAPPα and sAPPβ released into the culture medium under normoxic condition accounts for about 0.8, suggesting that both α- and β-secretory processing of endothelial APP occurs at the same extent. However, when ECs were subjected to hypoxic stress, the processing of endothelial APP is shifted toward the amyloidogenic route of APP cleavage, which may be due to (i) changes in enzyme activities of α- and/or β-secretases, and/or (ii) changes in the expression level of endothelial APP.

Hypoxic stress of EC for up to 48 hours of incubation resulted in enhanced generation and release of sAPPβ, as compared to corresponding control incubations, whereas the amount of sAPPα released into the culture medium did not change with incubation time, suggesting that hypoxic stress appears to predominantly affect the activity of the β-secretase, with presumably no consequences on the activity of the α-secretase. Similarly, incubation of SH-SY5Y cells under hypoxic conditions also affected APP processing but a reduced secretion of sAPPα into the culture medium has been observed.[35,36] Interestingly, similar observations have also been obtained by *in vivo* studies demonstrating that hypoxia affects APP metabolism by increasing expression and activity of BACE-1 and γ-secretase.[17,37,38]

On the other hand, hypoxic stress also induced the synthesis of new endothelial APP as revealed by Western analysis, which may explain the initial increase of both sAPPα and sAPPβ level in the culture medium of exposed EC. However, further exposure of hypoxic-stressed EC for up to 48 hours clearly indicates that the β-secretory route of APP processing dominates the α-secretory one. As the regulation of expression and synthesis of APP is highly complex and involves transcriptional, post-transcriptional, translational, and post-translational events including the action of regulatory RNA-binding proteins (for review, see[39]), more detailed investigations are required to disclose how hypoxic events may interfere with these molecular processes.

Regardless of that the question arises which factors and/or processes may contribute to the hypoxic stress-mediated induction of enhanced β-secretase activity in EC. Recently, we have shown in brain slices and primary neuronal cell cultures that VEGF may affect neuronal APP processing.[33,34] As VEGF was found to be up-regulated in EC by hypoxic stress, it was interesting to clarify whether VEGF plays also a role in controlling endothelial APP processing. While exposure of EC by VEGF hardly affected the amount of sAPPβ and Aβ(1–40) generated and secreted into the culture medium, the suppression of the VEGF receptor action by the VEGF receptor inhibitor SU-5416 resulted in decreased release of sAPPβ and Aβ(1–40), as compared to control incubations. These observations suggest that the hypoxic stress-mediated up-regulation of VEGF hardly contributes to the hypoxic stress-mediated changes in endothelial APP processing, but rather playing a counteracting role by maintaining a balanced APP processing. Moreover, the decreases in formation and secretion of Aβ(1–40) by suppression of the VEGF receptor action, suggest a role of VEGF in controlling the activity of γ-secretase via VEGF receptors, presumably via the VEGF receptor-associated tyrosine kinases. This suggestion is supported by a recent

study demonstrating that both receptor- and non-receptor tyrosine kinases may induce the cleavage of APP using an *in vitro* system based on the APP-Gal4-fusion protein stably transfected in SH-SY5Y neuroblastoma cell line.[40] However, the regulation of the γ-secretase, a multiprotein complex consisting of at least four distinct entities appears to be very complex, even due to the abundance of interacting partners and diversity of substrates.[41,42]

Hypoxic stress of EC also induced generation and secretion of nitric oxide (NO) which is certainly mediated by induction of the hypoxia-inducible factor (HIF)-1α transcription factor that subsequently may up-regulate iNOS expression followed by NO production.[43] HIF-1α, up-regulated in response to low level of cellular oxygen, is also known to increase BACE1 mRNA and protein level, which may provide one of the molecular mechanism by which hypoxic stress affects endothelial APP processing observed in this study.[38,44,45] On the other hand, a recent microdialysis study in rats reported a direct involvement of NO in up-regulation of hippocampal BACE1 expression and Aβ production following an acute ischemic episode.[46] The developmental temporal coincidence of increased levels of NO and reactive nitrogen species with the onset of Aβ plaque deposition in Tg2576 mice further suggests a role of NO in triggering expression and activity of β-secretase.[47] However, it remains to be elucidated whether the hypoxic stress-mediated increase in endothelial sAPPβ production represents a consequence of stress-induced NO formation.

Expression and activity of α- and β-secretases can be regulated on different levels of cell physiology such as transcription, translation, and post-translation,[48–51] while the molecular mechanisms controlling α- and β-secretase-cleavage of APP are not fully understood yet. However, signal molecules such as NO or VEGF may play a role in affecting those signaling pathways that mediate secretase activity beyond transcriptional and translational control, e.g. G-protein coupled receptor-, MAPK-, and tyrosine kinase signaling.

Hypoxic stress of EC resulted also in differential expression of p42/44 and P-p42/44 over the incubation time studied. Hypoxia-mediated up-regulation of p42/44 at a particular time of incubation was accompanied by a corresponding down-regulation of P-p42/44 suggesting the involvement of MAPK signaling in control and/or regulation of secretases. Indeed, there are a number of studies reporting links of MAPK signaling and expression and activity of both α- and β-secretases. In primary murine cortical neurons an up-regulation of the α-secretases (ADAM10) by activation of NMDA receptors via Wnt/MAPK signaling has been observed.[52] Another study in APPswe-transfected SH-SY5Y cells suggested a link of α-secretase activity and ERK signaling.[53] In PC12 cells, it was reported that the insulin-like growth factor 1-induced reduction of BACE1 expression might involve the MAPK/ERK1/2 signaling.[54] The activation of the ERK pathways induced the inhibition of α-, and γ-secretase activity,[55,56] as well as acts as negative modulator of BACE1 expression and activity.[57] Studies in SH-SY5Y cells suggested that formation of sAPPα and BACE1 expression are regulated by muscarinic acetylcholine receptors via ERK1/2.[58,59]

These experimental data fit very well with reports of changes in total ERK1/2 level observed in brain samples from AD patients as compared to healthy control subjects,[60–62] further supporting the important role of MAPK signaling in the pathogenesis of AD.

It is interesting to note that the hypoxic stress-mediated increase in formation and release of sAPPβ is accompanied by corresponding down-regulation of P-p42/44 expression. In Fig. 6 we have tentatively plotted the hypoxic stress-mediated increase in sAPPβ generation against the corresponding change in P-p42/44 expression, indicating a negative correlation of sAPPβ formation and P-p42/44 expression. These observations suggest that the activity of the β-secretase may be controlled and/or regulated by phosphorylation of p42/44.

Surprisingly, the exposure of EC to hypoxic stress affected neither the expression of phosphorylated nor of non-phosphorylated forms of JNK as compared to the corresponding normoxic controls, regardless of the incubation time examined. In contrast, 4-hydroxynonenal, an oxidative stress mediator has been observed to up-regulate BACE1 through the activation of JNK and p38-MAPK signaling,[51] suggesting

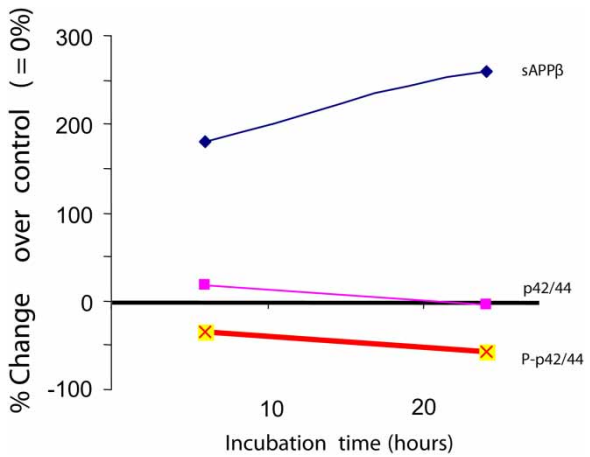

Figure 6 Correlation of hypoxic stress-mediated changes of sAPPβ and up-/down-regulation of p42/44 and P-p42/44 versus incubation time.

differential cellular mechanisms by which expression and activity of the β-secretase is driven.

In conclusion, the data presented in this study strongly suggest that hypoxic stress represents a mayor risk factor in mediating cerebral vascular Aβ accumulation by shifting the ratio of α- and β-secretory processing of endothelial APP toward the amyloidogenic route of APP metabolism. The enhancement of β-secretase activity as a consequence of hypoxic stress is presumably mediated by changing the phosphorylation status of the p42/44 MAPK, whereas the stress-induced up-regulation of VEGF appears to play a counteracting role by maintaining a balanced APP processing.

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